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In Vitro Estrogenicity of Polybrominated Diphenyl Ethers, Hydroxylated PBDEs, and Polybrominated Bisphenol A Compounds

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Polybrominated diphenyl ethers (PBDEs) are used in large quantities as additive flame retardants in plastics and textile materials. PBDEs are persistent compounds and have been detected in wildlife and in human adipose tissue and plasma samples. In this study, we investigated the (anti)estrogenic potencies of several PBDE congeners, three hydroxylated PBDEs (HO-PBDEs), and differently brominated bisphenol A compounds in three different cell line assays based on estrogen receptor (ER)-dependent luciferase reporter gene expression. In human T47D breast cancer cells stably transfected with an estrogen-responsive luciferase reporter gene construct (pEREtata-Luc), 11 PBDEs showed estrogenic potencies, with concentrations leading to 50% induction (EC_{50}) varying from 2.5 to 7.3 μM. The luciferase induction of the most potent HO-PBDE [2-bromo-4-(2,4,6-tribromophenoxy)phenol] exceeded that of estradiol (E_2), though at concentrations 50,000 times higher. As expected, brominated bisphenol A compounds with the lowest degree of bromination showed highest estrogenic potencies (EC_{50} values of 0.5 μM for 3-monobromobisphenol A). In an ERα-specific, stably transfected human embryonic kidney cell line (293-ERα-Luc), the HO-PBDE 4-(2,4,6-tribromophenoxy)phenol was a highly potent estrogen with an EC_{50} < 0.1 μM and a maximum 35- to 40-fold induction, which was similar to E_2. In an analogous ERβ-specific 293-ERβs-Luc cell line, the agonistic potency of the 4-(2,4,6-tribromophenoxy)phenol was much lower (maximum 50% induction compared to E_2), but EC_{50} values were comparable. These results indicate that several pure PBDE congeners, but especially HO-PBDEs and brominated bisphenol A-analogs, are agonists of both ERα and ERβ receptors, thus stimulating ER-mediated luciferase induction *in vitro.* These data also suggest that *in vivo* metabolism of PBDEs may produce more potent pseudoestrogens. *Key words*: ER-CALUX, estrogenicity, flame retardants, hydroxylated compounds, polybrominated diphenyl ethers. *Environ Health Perspect* 109:399–407 (2001). [Online 27 March 2001]
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Polybrominated diphenyl ethers (PBDEs) are widely used as additive flame retardants in many different polymers, resins, and substrates at concentrations ranging from 5% to 30% (*1*). Because of the widespread production and use of PBDEs, their high binding affinity to particles, and their lipophilic characteristics, several PBDE congeners bioconcentrate and bioaccumulate in the environment in a manner similar to the structurally related polychlorinated biphenyls (PCBs) (*1–3*). PBDEs have been detected in various biotic samples such as birds, seals, whales, and even in human blood, adipose tissue, and breast milk (*4–10*). The congeners 2,2′,4,4′-tetraBDE (BDE-47), 2,2′,4,4′,5-pentaBDE (BDE-99), and 2,2′,4,4′,6-pentaBDE (BDE-100) are generally the dominant congeners found in wildlife and humans. The relevance of PBDEs as environmental contaminants has been demonstrated by their accumulation in human breast milk, where concentrations in Swedish women have increased over the last 2 decades from 0.07 ng/g lipid weight in 1972 to 4.02 ng/g lipid weight in 1998 (*8*). Although PCB concentrations in wildlife are still higher than PBDE concentrations, they are declining over the same time period.

The most sensitive end points of PBDE toxicity *in vivo* are effects on thyroid function, observed as induction of thyroid hyperplasia and alteration of thyroid hormone production [i.e., lowering of free and total thyroxine (T_4) concentrations] in rats and mice (*11,12*). Consistent with these findings is the recent observation that several pure PBDE congeners were able to displace T_4 from transthyretin (TTR; a plasma transport protein of thyroid hormones) *in vitro*, after metabolic conversion to hitherto unidentified metabolites (*13*). These phenomena have also been observed for other organohalogen compounds such as PCBs and their hydroxylated metabolites (*14,15*, and references therein).

Another property that PBDEs share with PCBs and the polybrominated biphenyls (PBBs) is the dioxinlike, Ah receptor-mediated induction of cytochrome P450 1A1 and 1A2 *in vitro* (*16*) and *in vivo* (*17*). Recently we demonstrated by means of an Ah receptor-mediated, chemically activated luciferase expression cell line (the Ah-CALUX-assay) (*18–20*) that several pure di- to hepta-brominated PBDE congeners were able to act via this Ah receptor pathway *in vitro* as agonists and antagonists in a congener-specific manner (*21*). For example 2,3,4,4′,5,6-hexaBDE (BDE-166) and 2,3,3′,4,4′,5,6-heptaBDE (BDE-190) were relatively strong Ah receptor agonists with potencies comparable to the mono-*ortho* 2,3,3′,4,4′-pentaCB (CB-105) and 2,3′,4,4′,5-pentaCB (CB-118) (*22*).

Some studies have indicated that hydroxylated PBDEs (HO-PBDEs) are of potential environmental importance. In liver microsomes of rats, several PBDE congeners were biotransformed to metabolites (*13*). Örn and Klasson-Wehler (*23*) demonstrated that 2,2′,4,4′-tetraBDE (BDE-47) is biotransformed to HO-PBDEs in rats and mice. 3,5-Dibromo-2-(2,4-dibromophenoxy)phenol is a hydroxy-BDE that has been identified in blood plasma of Baltic salmon (*24*) at levels similar to those of the major PBDE congeners. Information on the endocrine activity of hydroxylated PBDEs is presently limited to the ability of several HO-PBDEs to bind competitively to the thyroid hormone receptor (*25*) and to TTR (*13*).

Studies showing that many industrial chemicals are weakly estrogenic compared to natural estrogens (*26–28*) have raised concern about their safety. For example, *o,p′*-DDT, bisphenol A, nonylphenol, and various phthalates possess estrogenic activity (*27*). The presumption is that these xenoestrogens may disrupt normal endocrine function, which can lead to reproductive failure

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and cancer of estrogen-sensitive tissues in humans and wildlife (*29*). Antiestrogenic activity by anthropogenic compounds has received less attention (*30*). Although the inhibition of hormone action and the resulting toxicological consequences have not been demonstrated conclusively, antiestrogenic action could critically affect sensitive reproductive and developmental processes as well (*30*). To date there have been no reports investigating the (anti)estrogenic activities of PBDEs and HO-PBDEs.

The aim of this study was to determine the (anti)estrogenicity of 17 PBDE congeners. We also examined three hydroxylated PBDEs that have halogen substitution patterns similar to those of thyroid hormones. The (anti)estrogenic activity of these compounds was tested *in vitro*, using an estrogen-responsive luciferase reporter cell line (T47D.Luc) (*31*). We compared the structure–activity relationships for (anti)estrogenicity of PBDE and HO-PBDE congeners with numerous other brominated flame retardants, such as differently brominated bisphenol A compounds. We also tested the most potent PBDEs and HO-PBDEs observed in T47D.Luc cells for estrogen receptor specificity using 293 human embryonic kidney cells stably transfected with recombinant human estrogen receptor (ERα or ERβs) cDNA and the luciferase reporter gene construct (*32–34*).

Materials and Methods

Chemicals. The 17 PBDE congeners (> 98% pure; Figure 1, Table 1) were synthesized as described earlier (*35,36*). Three HO-PBDEs, 4-(2,4,6-tribromophenoxy)phenol (T_2-like HO-BDE), 2-bromo-4-(2,4,6-tribromophenoxy)phenol (T_3-like HO-BDE), and 2,6-dibromo-4-(2,4,6-tribromophenoxy)phenol (T_4-like HO-BDE) (Figure 1) were synthesized as described by Marsh et al. (*25*) and were at least 99% pure. We use the abbreviations for these HO-PBDEs (T_2-like, T_3-like-, T_4-like HO-BDE) according to their resemblance in halogen substitution patterns to the thyroid hormones 3,5-diiodothyronine (3,5-T_2), 3,3´,5-triiodothyronine (T_3), and 3,3´,5,5´-tetraiodothyronine (T_4). The core structure of PBDEs and the structures of the HO-PBDEs used in this study are shown in Figure 1, including the structure of the analog 4-phenoxyphenol. The numbering system for individual PBDE congeners is based on the numbering system applied to PCBs (*37*). 4-Phenoxy-phenol and bisphenol A were obtained from Aldrich Chemical Company (Bornem, Belgium). 17β-Estradiol (E_2; 99%) and ethanol (100%, pro analysis) were purchased from Sigma Chemical Company (St. Louis, MO, USA). ICI 182,780 was a gift from A. Wakeling, Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK). 3-Monobromobisphenol A (MBBPA; 96.5% pure, with 3.5% 3,3´-dibromobisphenol A), 3,3´-dibromobisphenol A (diBBPA; 99.4% pure, with 0.6% 3,3´,5-tribromobisphenol A), and 3,3´,5-tribromobisphenol A (triBBPA; 100% pure) were synthesized by bromination of bisphenol A using bromine in acetic acid at room temperature. The test chemicals and E_2 were dissolved in ethanol or dimethyl sulfoxide (DMSO; 99.9% pure, Janssen Chimica, Geel, Belgium) for use in the *in vitro* assays.

Cell culture. We used the human T47D breast cancer cell line stably transfected with an estrogen-responsive luciferase reporter gene construct (pEREtata-Luc) (*31*) to study the *in vitro* (anti)estrogenic activity of PBDEs and HO-PBDEs. The T47D.Luc cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's (DMEM) medium and Ham's F12 (DF) medium (Gibco Brl, Life Technologies, Breda, The Netherlands) supplemented with sodium bicarbonate, nonessential amino acids, sodium pyruvate, and 7.5% fetal calf serum (heat inactivated) at 37°C and 7.5% CO_2.

The preparation of the stably transfected 293-Luc cell lines (ERα and ERβs) has been described in detail elsewhere (*32*). Briefly, human 293 embryonal kidney (HEK) cells (ATCC, American Type Culture Collection, Rockville, MD, USA) were stably transfected with the pEREtata-Luc construct (*31,32*) cotransfected with an antibiotic resistance gene. This cell line was subsequently transfected with a recombinant

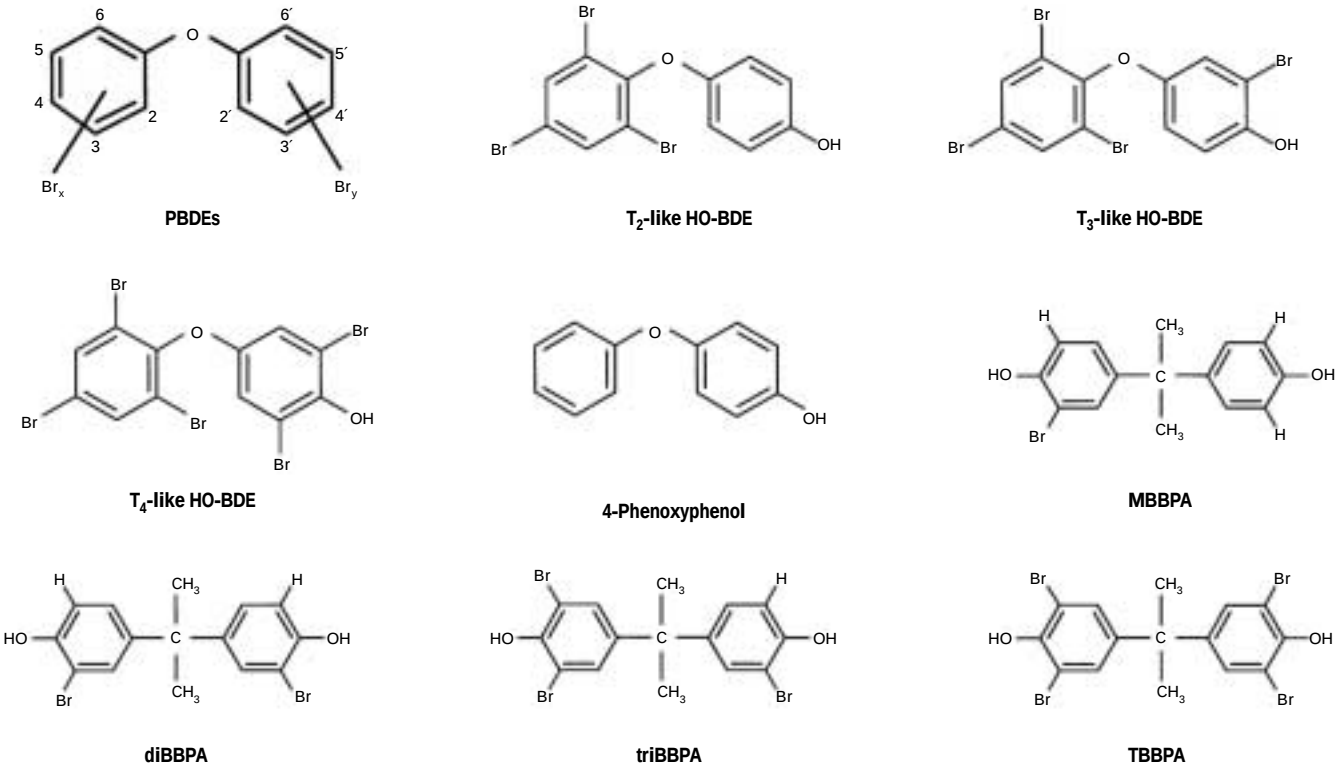

Figure 1. Structure of PBDEs, the three hydroxylated PBDEs, 4-phenoxyphenol, and the differently brominated bisphenol A analogs. The hydrogens have been omitted for clarity.

human estrogen receptor (ERα or a short form of ERβ, ERβs) cDNA and a different antibiotic resistance gene. The 293-ERα- and 293-ERβs-Luc cell lines were cultured in a 1:1 mixture of DMEM and DF medium supplemented with 7.5% fetal calf serum (heat inactivated) at 37°C and 7.5% CO_2.

ER-CALUX assay. We performed the T47D.Luc-based assay as described previously (*31*). The cells were trypsinized, resuspended in assay medium, and seeded in 96-well plates (Packard, Meriden, CT, USA) at a density of 5,000 cells per well in 100 µL. The assay medium consisted of phenol red-free DF and fetal calf serum treated with 5% dextran-coated charcoal (DCC-FCS). DCC-FCS was prepared as described by Horwitz and McGuire (*38*). After 24 hr, when wells were approximately 50% confluent, the assay medium was renewed. After another 24 hr, the assay medium was replaced by incubation medium (for preparation, see below), containing DMSO or ethanol stock solutions of the test compounds or estradiol. Solvent concentrations did not exceed 0.1%. The incubation medium was removed after an incubation of 24 hr at 37°C in an atmosphere of 7.5% CO_2. Cells were washed twice with 100 µL phosphate-buffered saline (PBS) and subsequently lysed in 30 µL low salt (LS) buffer containing 10 mM Tris (pH 7.8), 2 mM dithiothreitrol (DTT), and 2 mM 1,2-diaminocyclohexane-*N,N,N´,N´*-tetraacetic acid. After 10 min of incubation on ice, the 96-well plates were frozen at –80°C for a minimum of 30 min and maximum of 1 day to lyse the cells. The plates were thawed on ice and shaken for 5 min at room temperature. We measured luciferase activity in a luminometer (Labsystems Luminoscan RS, Breda, The Netherlands) with automatic injection of 100 µL flash mix (pH 7.8) per well containing 470 µM luciferin, 20 mM trycine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 5 mM ATP, and 2 mM DTT (pH 7.8).

293-ERα- and 293-ERβs-Luc assay. The 293-ERα- and 293-ERβs-Luc-based assays were performed similarly to the ER-CALUX assay and have been described previously (*32–34*). Briefly, cells were trypsinized and resuspended in assay medium composed of phenol red-free DF containing 30 nM selenite, 10 µg/mL transferin, and 0.2% BSA supplemented with 5% DCC-FCS. The cells were seeded in 96-well plates at a density of 15,000 cells per well in 200 µL assay medium. After 48 hr the cells were 50–60% confluent, and the assay medium was replaced by incubation medium (i.e., containing a 1,000-fold dilution of test compounds) as described for the ER-CALUX assay. After an incubation of 24 hr at 37°C in an atmosphere of 7.5% CO_2, the plates were transferred to ice and the medium was removed by suction. Luciferase production was assayed as described above for the ER-CALUX assay.

Exposure of cells. Before the T47D.Luc cell incubations, the PBDE and HO-PBDE stock solutions (prepared in DMSO) and the brominated bisphenol compounds (prepared in ethanol) were diluted 1,000-fold in assay medium in a 48-well plate (to obtain a solvent-concentration of 0.1% v/v) and thoroughly shaken, and 100 µL was added to the cells in 96-well plates. The nominal concentrations of the toxicants in the medium were 0.05, 0.1, 0.5, 1.0, and 5 µM, and for potent compounds concentrations of 2.5 and 10 µM were also included. For each experiment, we included a complete E_2 standard curve (1–100 pM, 7 different concentrations in total). In addition, we tested three calibration points (0, 10, and 30 pM E_2) on every 96-well plate within an experiment.

For the 293-ERα- and 293-ERβs-Luc assays, the DMSO stock solutions of the tested compounds were diluted 1,000-fold in the appropriate assay medium. The nominal concentrations of the toxicants exposed to the cells were 1.0, 5.0, and 10 µM. For each experiment a complete E_2 standard curve (0.001–10,000 pM in eight different concentrations) was included. For all three ER-CALUX assays, we tested every toxicant concentration in triplicate and repeated each assay at least twice.

Antiestrogenic effects. We tested the possible antiestrogenic effects of the compounds in the ER-CALUX assay at the same nominal concentrations as for the estrogenic activity screening. The T47D.Luc cells were coincubated with an E_2 concentration of 10 pM. This E_2 concentration was the approximate EC_{50} for the induction of luciferase activity (*31*). The percentage (v/v) of DMSO present during these antiestrogenicity incubations was 0.2%. An antiestrogenic effect in this assay was defined by the capacity of a chemical to inhibit the luciferase activity induced by the approximate EC_{50} concentration of E_2. The percentage inhibition is calculated according to the equation

Table 1. Estrogenic activity of polybrominated diphenyl ethers (PBDEs), hydroxylated PBDEs (HO-PBDEs), and brominated bisphenols in the ER-CALUX assay with T47D.Luc cells.

Compound	Bromine substitution	LOEC (µM)[a]	Relative potency (LOEC)[b]	EC_{50} (µM)[c]	Relative potency (EC_{50})[d]	Maximum luciferase induction (%)[e]
Estradiol		1.0×10^{-6}	—	1.0×10^{-5}	—	100
PBDEs						
BDE-15	4,4´	NA	—	—	—	< 1
BDE-28	2,4,4´	0.5	2.0×10^{-6}	NA	—	43 ± 2
BDE-30	2,4,6	0.5	2.0×10^{-6}	3.4	2.9×10^{-6}	114 ± 31
BDE-32	2,4´,6	0.05	2.0×10^{-5}	5.1	1.9×10^{-6}	85 ± 13
BDE-47	2,2´,4,4´	5.0	2.0×10^{-7}	NA	—	6 ± 1
BDE-51	2,2´,4,6´	0.5	2.0×10^{-6}	3.1	3.2×10^{-6}	85 ± 18
BDE-71	2,3´,4´,6	0.5	2.0×10^{-6}	7.3	1.4×10^{-6}	62 ± 8
BDE-75	2,4,4´,6	0.5	2.0×10^{-6}	2.9	3.5×10^{-6}	53 ± 10
BDE-77	3,3´,4,4´	NA	—	—	—	< 1
BDE-85	2,2´,3,4,4´	5.0	2.0×10^{-7}	NA	—	8 ± 1
BDE-99	2,2´,4,4´,5	5.0	2.0×10^{-7}	NA	—	2 ± 1
BDE-100	2,2´,4,4´,6	0.05	2.0×10^{-5}	2.5	4.1×10^{-6}	57 ± 10
BDE-119	2,3´,4,4´,6	0.05	2.0×10^{-5}	3.9	2.6×10^{-6}	25 ± 4
BDE-138	2,2´,3,4,4´,5´	NA	—	—	—	1 ± 1
BDE-153	2,2´,4,4´,5,5´	NA	—	—	—	< 1
BDE-166	2,3,4,4´,5,6	NA	—	—	—	< 1
BDE-190	2,3,3´,4,4´,5,6	NA	—	—	—	< 1
HO-PBDEs						
4-Phenoxyphenol	0.5	2.0×10^{-6}	1.7	5.8×10^{-6}		195 ± 17
T_2-like HO-BDE	0.05	2.0×10^{-5}	0.1	1.0×10^{-4}		160 ± 11
T_3-like HO-BDE	0.5	2.0×10^{-6}	0.5	2.0×10^{-5}		119 ± 22
T_4-like HO-BDE	NA	—	—	—		< 1
(Brominated) bisphenols						
Bisphenol A	0.01	1.0×10^{-4}	0.3	3.3×10^{-5}		200 ± 15
MBBPA	0.1	1.0×10^{-5}	0.5	2.0×10^{-5}		125 ± 3.1
DiBBPA	0.1	1.0×10^{-5}	0.4	2.5×10^{-5}		136 ± 1
TriBBPA	0.5	2.0×10^{-6}	> 10	$< 1.0 \times 10^{-6}$		80 ± 3
TBBPA	NA	—	—	—		< 1

NA, Not achieved.
[a]Lowest observed effect concentration; lowest concentration where luciferase activity is detected. [b]Ratio between dose of compound and estradiol needed to achieve an estrogenic effect [$LOEC_{(E2)}$ / $LOEC_{(compound)}$]. [c]Concentration at which the induction of luciferase activity is 50% of the maximum. [d]Ratio between EC_{50} of the compound and EC_{50} of estradiol. [e]Percent luciferase activity induced by the test compound relative to the maximum luciferase activity of E_2 (30 pM). Maximum concentration of the test compounds was 10 µM, except for BDE-47 and BDE-85 (maximum: 5 µM).

$$I(\%) = 100\left(1 - \frac{L_{test} - L_{control}}{L_{E2} - L_{control}}\right) \quad [1]$$

where I is the percent inhibition, and L_{test}, $L_{control}$, and L_{E2} are the average luciferase activity of three test wells, three control wells and six wells incubated with 30 pM of E_2, respectively. Using Equation 1, a compound without antagonistic activity will show the same luciferase induction as 10 pM of E_2, [i.e., 63.3 ± 7.5% (see "Results")]. On each plate a positive control of 10 nM of the competitive ER antagonist ICI 182,780 was included in triplicate. ICI 182,780 produces virtually total antagonism of E_2-induced luciferase activity at this concentration [i.e., activity measured is equal to solvent control levels (*31*)].

Cytotoxicity. We measured possible cytotoxic effects of the tested compounds in the bioassays using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide activity (*39*). To determine cytotoxic effects, we seeded cells and exposed them to the test compounds in the same manner as outlined in their corresponding assay procedures.

Dose–response curves and statistics. Possible dose–response relations were described by the sigmoidal function

$$y = a_0 + a_1/\{1 + \exp[(a_2 - x)/a_3]\} \quad [2]$$

using SlideWrite Plus 4.0 (Advanced Graphics Software, Carlsbad, CA, USA), where y is the induction of luciferase activity compared to controls for estrogenic effects, or inhibition [I (%), Equation 1] for antiestrogenic effects, x is the logarithm of the dose, and a_1 is the maximum y-value. We tested the significance of the data fits using one-way analysis of variance at $p < 0.05$.

Results

Cytotoxicity

In the concentration range of 0.01–10 µM, none of the incubations of the PBDEs or HO-PBDEs showed any significant effect on MTT activity relative to the solvent control (data not shown). Furthermore, no cytotoxic effect could be observed by microscopic examination in this concentration range.

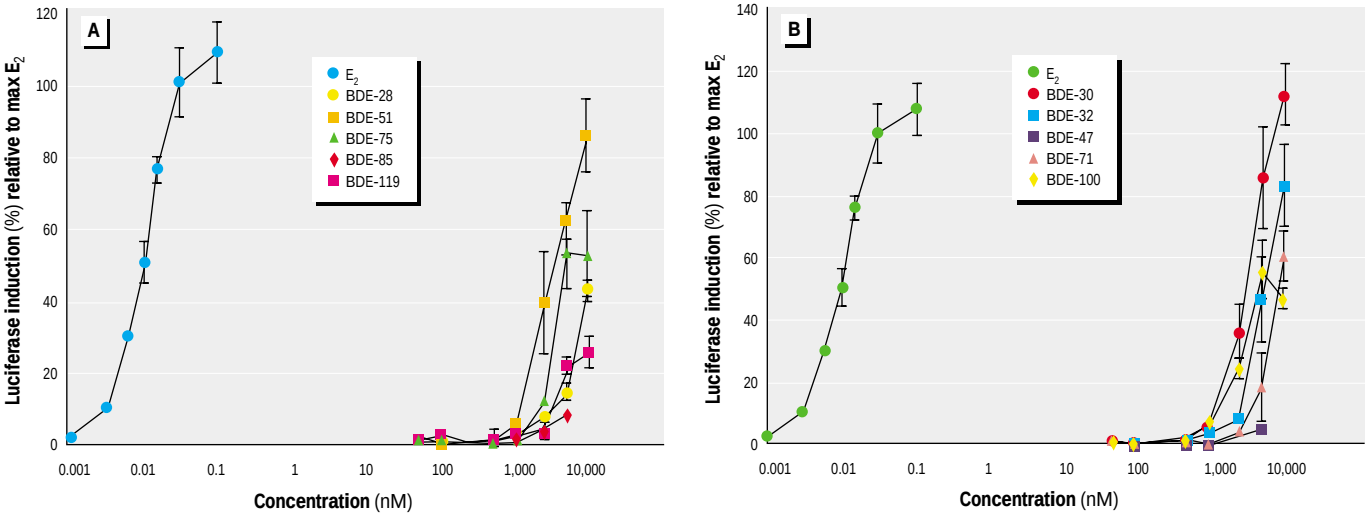

Figure 2. Estrogenic activity of PBDEs in the T47D.Luc cells. Luciferase induction (%) relative to the maximum induction by E_2 (30 pM) after 24-hr exposure to several concentrations of (*A*) BDE-28, -51, -75, -85 and -119, and (*B*) BDE-30, -32, -47, -71, and -100. Points are means (n = 3) ± SD (bars) for each concentration.

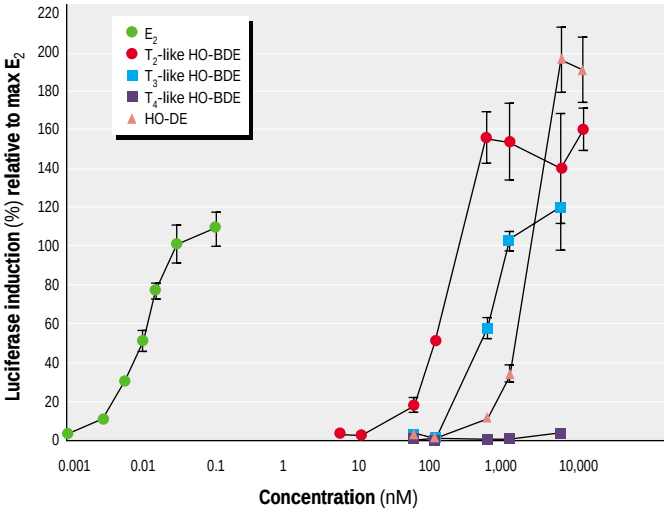

Figure 3. The estrogenic activity of hydroxy-PBDEs in the T47D.Luc cells. Luciferase induction (%) relative to the maximum induction by E_2 (30 pM) after 24-hr exposure to several concentrations of T_2-like HO-BDE [4-(2,4,6-tribromophenoxy)phenol], T_3-like HO-BDE [2-bromo-4-(2,4,6-tribromophenoxy)phenol], T_4-like HO-BDE [2-6-dibromo-4-(2,4,6-tribromophenoxy)phenol], and 4-phenoxyphenol. Points are means (n = 3) ± SD(bars) for each concentration.

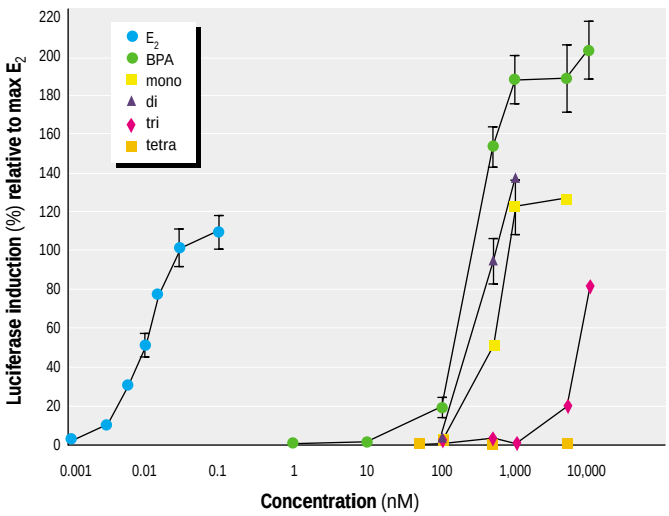

Figure 4. The estrogenic activity of differently brominated bisphenols in the T47D.Luc cells. Luciferase induction (%) relative to the maximum induction by E_2 (30 pM) after 24-hr exposure to several concentrations of bisphenol A (BPA), monobromobisphenol A (mono), dibromobisphenol A (di), tribromobisphenol A (tri), and tetrabromobisphenol A (tetra). Points are means (n = 3) ± SD (bars) for each concentration.

PBDE concentrations could not exceed 10 µM because of solubility problems and slight cytotoxic effects (data not shown).

ER-CALUX Assay Based on T47D.Luc Cells

Estrogenic effects. Seventeen PBDE congeners and 3 HO-PBDEs were tested in the T47D.Luc-based ER-CALUX assay for their estrogenic and/or antiestrogenic properties. Eleven PBDEs exhibited luciferase induction (Table 1) in a dose-dependent manner (Figure 2). The most potent PBDE-congeners [2,2´,4,4´,6-pentaBDE (BDE-100) > 2,4,4´,6-tetraBDE (BDE-75) > 2,2´,4,6´-tetraBDE (BDE-51) > 2,4,6-triBDE (BDE-30) > 2,3´,4,4´,6-pentaBDE (BDE-119)] showed EC_{50} values within a small concentration range of 2.5 to 3.9 µM (Table 1). These PBDE agonists were 250,000–390,000 times less potent than the natural ligand, E_2.

The T_4-like HO-BDE compound demonstrated no estrogenic effect up to 10 µM (Figure 3). In contrast, the T_3-like HO-BDE and T_2-like HO-BDE showed the highest estrogenic potencies (EC_{50} 0.5 and 0.1 µM, respectively) among all compounds tested in this study (Table 1, Figure 3). The compound 4-phenoxy-phenol was included for comparison because it is structurally analogous to the hydroxylated PBDEs. The T_2-like and T_3-like HO-BDEs induced maximum luciferase activity at 0.5 µM and 1.0 µM respectively, and this maximum luciferase activity (160 ± 11 and 119 ± 22 %) exceeded that of the natural hormone E_2 (Table 1).

Of the brominated bisphenols tested, MBBPA and diBBPA showed estrogenic activities comparable to the T_3-and T_2-like HO-BDE, with EC_{50} values of 0.5 and 0.3 µM, respectively (Figure 4, Table 1). The maximum luciferase activity of bisphenol A, MBBPA, and diBBPA exceeded the maximum activity induced by E_2 (Figure 4). Bisphenol A and 4-phenoxyphenol had the highest maximum luciferase activity of 199 ± 15% and 195 ± 17%, respectively, relative to the maximum of E_2 (set at 100%, Figure 4). Tetrabromobisphenol A (TBBPA) showed no estrogenic potency within the tested concentrations.

Antiestrogenic effects. The antiestrogenic potency of PBDEs was determined in the ER-CALUX bioassay by treating T47D.Luc cells with 0.01 to 10 µM concentrations of PBDEs in the presence of 10 pM of E_2. Alone, this E_2 concentration produced a luciferase induction of 63.3 ± 7.5% of the maximum (Table 2). At the 10 nM concentration, the ER antagonist ICI 182,780 completely inhibited the luciferase activity induced by 10 pM E_2. Only 2,2´,4,4´,5,5´-hexaBDE (BDE-153), 2,3,4,4´,5,6-hexaBDE (BDE-166), and 2,3,3´,4,4´,5,6-hepta-BDE (BDE-190), which did not induce luciferase activity alone (up to 10 µM, Table 1), reduced E_2-induced luciferase activity (Table 2). Moreover, these three PBDE congeners inhibited the E_2-induced activity in a dose-dependent manner (Figure 5).

293-ERα- and 293-ERβs-293-Luc Cell Lines

As in previous findings (*32–34*), the luciferase activity for the 293-ERα-Luc assay was more sensitive and responsive to E_2 than the 293-ERβs-Luc assay (data not shown). In the present study, the 293-ERα-Luc assay had a 35-fold maximum induction relative to control, which was reached at about 100 pM E_2. The lowest observed effect concentration (LOEC) and EC_{50} for E_2 in the 293-ERα-Luc assay were 2.6 pM and 11.9 pM, respectively. In the 293-ERβs-Luc assay, a 16-fold maximum induction was attained at about 1,000 pM E_2. The LOEC was 15.3 pM and the EC_{50} was 90.2 pM for E_2.

The most potent xenoestrogens in the ER-CALUX—BDE-30, BDE-100, 4-phenoxy-phenol, and T_2-like HO-BDE—were investigated for estrogenicity in the 293-ERα- and ERβs-Luc-based assays. Relative to the E_2 maximum luciferase induction, the induction of the highest concentration of BDE-30 (10 µM) in the 293-ERα-Luc and 293-ERβs-Luc cell lines (34.2 ± 2.2% and 7.8 ± 3.1%, respectively; Figure 6A,B) was much lower compared to the T47D.Luc cell line (114 ± 31%). At the same concentration,

Table 2. Antiestrogenic activity of PBDEs and HO-PBDEs in combination with 10 pM E_2 in the ER-CALUX assay with T47D.Luc cells.

Compound	Bromine substitution	Concentration (µM)	IC_{50} (µM)[a]	Percent luciferase induction relative to maximum E_2 (30 pM)[b]
Estradiol		1.0×10^{-5}	—	63.3 ± 7.5
ICI 182,780		0.01	1.0×10^{-5}	0 ± 1.2
PBDEs				
BDE-15	4,4´	5	—	62.1 ± 1.5
BDE-28	2,4,4´	0.5	—	80.1 ± 12.9
		5.0	—	111.5 ± 3.3
BDE-30	2,4,6	0.5	—	31 ± 16
		5.0	—	47 ± 10
BDE-32	2,4´,6	0.5	—	64.8 ± 6.4
		5.0	—	106.8 ± 4.0
BDE-47	2,2´,4,4´	0.5	—	45.9 ± 5.9
		5.0	—	75.0 ± 4.8
BDE-51	2,2´,4,6´	0.5	—	108.0 ± 8.2
		5.0	—	95.0 ± 4.8
BDE-71	2,3´,4´,6	0.5	—	52 ± 6
		5.0	—	62 ± 1
BDE-75	2,4,4´,6	0.5	—	41.9 ± 7.6
		5.0	—	55.1 ± 12.6
BDE-77	3,3´,4,4´	5.0	—	64.9 ± 2.2
BDE-85	2,2´,3,4,4´	5.0	—	68.9 ± 2.9
BDE-99	2,2´,4,4´,5	5.0	—	62.8 ± 2.2
BDE-100	2,2´,4,4´,6	0.5	—	86.6 ± 3.7
		5.0	—	108.0 ± 15.4
BDE-119	2,3´,4,4´,6	0.5	—	102.6 ± 5.4
		5.0	—	92.5 ± 12.7
BDE-138	2,2´,3,4,4´,5´	5.0	—	56.9 ± 6.6
BDE-153	2,2´,4,4´,5,5´	0.5	3.1	67.9 ± 3.4
		5.0		47.4 ± 9.2
BDE-166	2,3,4,4´,5,6	0.5	0.8	52.4 ± 4.1
		5.0		25.3 ± 4.9
BDE-190	2,3,3´,4,4´,5,6	0.5	1.0	74.1 ± 5.8
		5.0		43.8 ± 3.7
HO-PBDEs				
4-Phenoxyphenol		0.5	—	67.0 ± 9.6
		5.0	—	209.3 ± 25.7
T_2-like HO-BDE		0.5	—	169.4 ± 14.8
		5.0	—	178.5 ± 13.1
T_3-like HO-BDE		0.5	—	82.6 ± 7.6
		5.0	—	96.1 ± 12.2
T_4-like HO-BDE		0.5	—	62.1 ± 5.2
		5.0	—	65.1 ± 13.8

[a]Concentration at which the induction of luciferase activity by E_2 (EC_{50} concentration of 10 pM) is inhibited by 50%. [b]The luciferase activity induced by the test compound and E_2 (EC_{50} concentration of 10 pM) as a percentage of the maximum activity (E_2, 30 pM).

BDE-100 showed an induction < 2% in the 293-ERβs-Luc assay, whereas the 293-ERα-Luc assay was more responsive (about 20% relative induction). However, EC_{50} values of BDE-30 and BDE-100 in the 293-ERα-Luc-assay (< 5.0 µM) and the ER-CALUX assay (3.4 and 2.5 µM, respectively) are comparable.

The T_2-like HO-BDE induced maximum response in the 293-ERα-Luc cells to the same maximum level as E_2 (Figure 6A), even at the lowest concentration tested (0.1 µM). With the 293-ERβs-Luc assay, the T_2-like HO-BDE showed 50% of the maximum E_2 induction at 5.0 µM (Figure 6B). The structural analog 4-phenoxyphenol induced luciferase in the 293-ERβs-Luc to 126 ± 12 and 144 ± 11% at concentrations of 5.0 µM and 10 µM, respectively (Figure 6B). The luciferase induction by 4-phenoxyphenol in the 293-ERα-Luc assay similarly exceeded the maximal E_2 induction (Figure 6A), but only at the 10 µM concentration level. The EC_{50} value of this compound in the 293-ERα- and 293-ERβs-Luc assays is in the same range (< 5.0 µM).

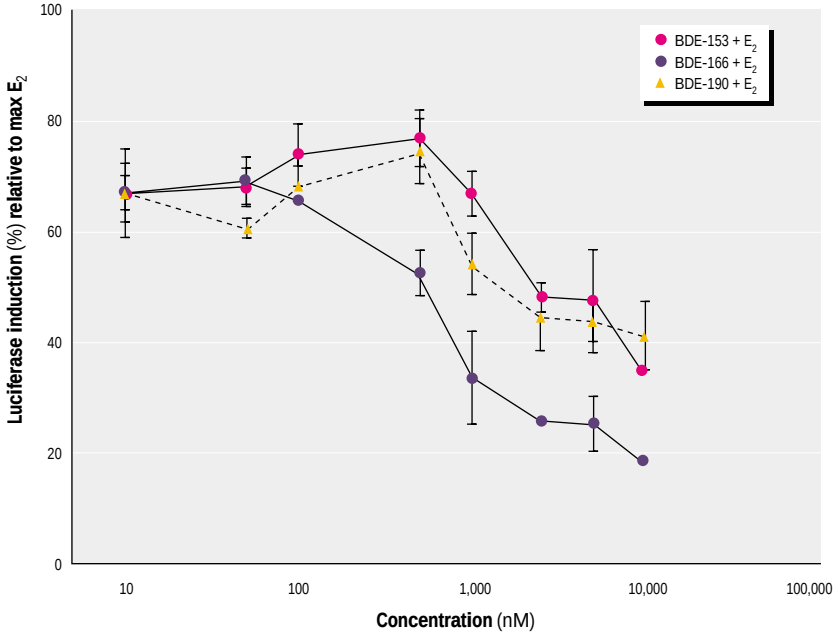

Figure 5. The antiestrogenic activity of PBDEs in the T47D.Luc cells. The luciferase induction (%) relative to the maximum induction by E_2 (30 pM) after 24-hr exposure to several concentrations of BDE-153 (2,2′,4,4′,5,5′-hexaBDE), BDE-166 (2,3,4,4′,5,6-hexaBDE), and BDE-190 (2,3,3′,4,4′,5,6-heptaBDE), in the presence of 10 pM E_2 (with luciferase induction of 63.3 ± 7.5% of the maximum induction). Points are means (n = 3) ± SD (bars) for each concentration.

Discussion

In this study we investigated both the estrogenic and antiestrogenic activity of several PBDE congeners, three hydroxylated PBDEs, and some brominated bisphenol A compounds *in vitro*. To our knowledge, no studies have been performed on the agonistic or antagonistic activity of these compounds *in vivo* or *in vitro* at the level of the estrogen receptor. Of the 17 selected PBDEs, 11 congeners were able to exert estrogenic activities in T47D.Luc cells at LOECs as low as 0.05 µM, and EC_{50} values ranging from 2.5 to 7.3 µM. In the same ER-CALUX assay, the organochlorine pesticides methoxychlor, endosulfan, and chlordane had a similar potency for luciferase induction, about 1.0×10^{-6} times the potency of E_2 (*31*).

PBDE congeners with the highest estrogenic activity in the T47D.Luc cells were 2,2′,4,4′,6-pentaBDE (BDE-100), 2,4,4′,6-tetraBDE (BDE-75), and 2,2′,4,6′-tetraBDE (BDE-51). BDE-100 in particular has often been reported among the more common PBDEs found in humans and other mammals (*2*,*7*,*9*). The common structural features among the estrogenic PBDEs are two *ortho* (2,6)-bromine atoms on one phenyl ring, at least one *para*-bromine atom (preferably on the same phenyl-ring as the *ortho* bromines), and nonbrominated *ortho-meta* or *meta* carbons on the other phenyl ring. This structure–activity relationship resembles the one suggested by Korach et al. (*40*) for hydroxylated PCBs in a competitive binding assay, where congeners with the highest binding affinity for the estrogen receptor contained an unsubstituted phenol ring with a *p*-hydroxy group (e.g., 4-hydroxy-2′,4′,6′-triCB). However, in the case of the brominated diphenyl ethers, the

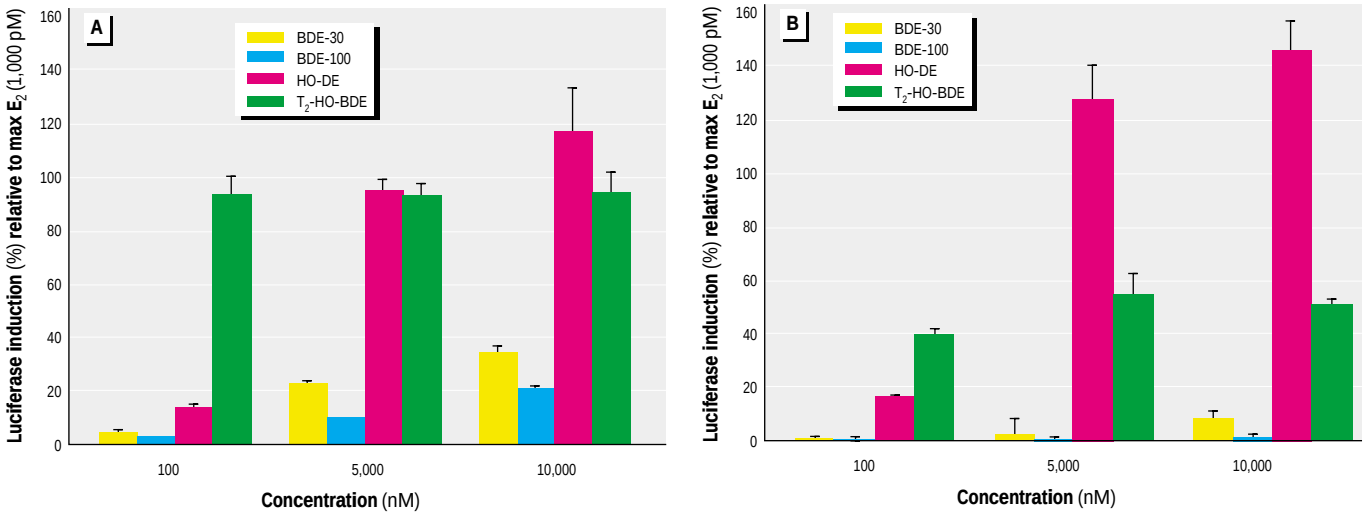

Figure 6. The estrogenicity of PBDEs and HO-PBDEs in the (*A*) 293-ERα-Luc and (*B*) 293-ERβs-Luc cells. The luciferase induction (%) relative to the maximum induction by E_2 (1,000 pM) after 24-hr exposure to three concentrations of BDE-30 (2,4,6-triBDE), BDE-100 (2,2′,4,4′,6-pentaBDE), T_2-like HO-BDE [4-(2,4,6-tribromophenoxy)phenol], and HO-DE (4-phenoxyphenol). Points are means (n = 3) ± SD (bars) for each concentration.

para position is occupied by a bromine atom. In addition, Connor et al. (*41*) observed that hydroxy-PCB congeners having one or no chlorine atoms *ortho* to the *para*-hydroxyl group and 2,4,6-trichlorination on the nonphenolic ring induced luciferase activity from 20 to 60% (at 5 µM) of the E_2 maximum induction in HeLa.Luc cells. Introduction of a single 2- or 3-chloro substituent into the phenolic ring significantly decreased the ER binding affinity (*41*). In case of PBDEs, the introduction of a single 3-bromine substituent next to the *para*-bromine atom on one ring significantly reduced the estrogenic potency [e.g., EC_{50}-value of 2,4,4´,6-tetraBDE (2.9 µM) is lower compared to 2,3´,4,4´,6-pentaBDE (3.9 µM)], whereas the introduction of a single 2-bromine (*ortho*) substituent next to the *para*-bromine increased the estrogenic potency, though not significantly (EC_{50} value of 2,2´,4,4´,6-pentaBDE: 2.5 µM).

Though not very likely, hydroxylated PBDE metabolites formed *in situ* may have been involved in the estrogenic effects of the PBDEs in the T47D.Luc cells. T47D.Luc cells possess some metabolic capabilities such as cytochrome P450-mediated hydroxylation of estrogens and xenobiotics. P450 1A (*42,43*), P450 1B (*43*), and 17β-hydroxysteroid dehydrogenase (*44*) have been reported in T47D.Luc cells. However, few data are available on the metabolism of PBDEs, and in the only two studies known reporting PBDE metabolism, the major compound excreted was the parent PBDE. Örn and Klasson-Wehler (*23*) detected five hydroxylated PBDE metabolites (by GC/MS analyses) in feces and various tissues of rats and mice dosed orally with 2,2´,4,4´-tetraBDE (BDE-47), but the major compound excreted was BDE-47. Larsen et al. (*45*) reported a low (about 1% of the total given dose) biliary and urinary excretion of possible metabolites of 2,2´,4,4´,5-pentaBDE (BDE-99) in conventional and bile-duct cannulated rats.

The hydroxylated PBDE congeners tested in our study have structural resemblance with the thyroid hormones 3,5-diiodothyronine (T_2), 3,3´,5-triiodothyronine (T_3), and 3,3´,5,5´-tetraiodothyronine (thyroxine, T_4). These HO-PBDEs have been reported to bind to the human α- and β-thyroid hormone receptor (THR) (*25*) and compete with the natural hormone T_4 for binding to a human thyroid hormone transport protein, transthyretin (*13*) *in vitro*. Several interactions between the thyroid hormone receptor- and estrogen receptor-mediated pathways have been reported, affecting testis development (*46*) and behavior (*47*). Since the structure of the hydroxy-phenyl ring in compounds interacting with the ER and THR (hydroxylated PCBs, hydroxylated PBDEs) is similar (with differences in halogen substitution), it is interesting to study the possible interaction of compounds with both pathways. The ranking of estrogenic potency in the T47D.Luc cells of the thyroid hormonelike HO-PBDEs was T_2-like HO-BDE (EC_{50}, 0.1 µM) > T_3-like HO-BDE (EC_{50}, 0.5 µM) >>> T_4-like HO-BDE. The potencies of the T_2-like HO-BDE and T_3-like HO-BDE were virtually the same as the potency of the phenolic industrial chemicals, such as bisphenol A [0.3 µM (this study), 0.8 µM (*31*)] and nonylphenol [0.3 µM (*31*)] tested in the same ER-CALUX assay. Bisphenol A is one of several well-defined phenolic environmental estrogens that are known to elicit estrogen-mediated responses *in vivo* and *in vitro* such as the increased proliferation of MCF7 human breast cancer cells (*48 –51*). The ranking order for estrogenicity of the hydroxylated PBDEs (Figure 3) was the reverse order found for binding to the human α- and β-thyroid hormone receptor (THR) (*25*) and human transthyretin (TTR) (*13*) *in vitro*. This comparison between ER and THR interactions emphasizes that nonbromination of the phenolic ring is necessary for optimum interaction with the ER, which was also found for HO-PCBs (*40,41*). Conversely, like the interaction of the natural, iodine-containing T_2, T_3, and T_4 thyroid hormones with THR and TTR, increasing bromination in adjacent positions on the HO-PBDEs increases THR and TTR binding affinity. The same is true for the brominated bisphenols. The ranking of estrogenic potency in the T47D.Luc cells of the brominated bisphenols was monoBBPA (EC_{50}, 0.5 µM) ~ diBBPA (EC_{50}, 0.3 µM) >> triBBPA (EC_{50} > 10 µM) >>> TBBPA, and was also the reverse order found for interaction with human TTR *in vitro* (*13*). The addition of bromine atoms in the *meta* position of the aromatic ring (in diBBPA) had no significant effect on the estrogenic potency. This is in line with results published by Perez et al. (*51*), where the estrogenicity of 2,2-bis(4-hydroxy-3-methylphenyl)propane (i.e., one methylgroup in the *meta* position of one aromatic ring) in a bioassay with MCF7 human breast cancer cells was not changed compared to bisphenol A. However, the introduction of two bromine atoms in the *meta* position of one aromatic ring drastically decreased the estrogenic potency (triBBPA, this study).

In contrast to the HO-PBDEs, the major HO-PCBs identified in human serum were mostly antiestrogenic but exhibited low to nondetectable estrogenic activities in several *in vitro* bioassays (*48*). At concentrations as high as 10 M, several 4-OH-substituted PCBs were not estrogenic toward binding of rat uterine ER. Furthermore, the same HO-PCBs did not induce the proliferation of MCF7 human breast cancer cells, or the luciferase activity of transiently transfected HeLa.Luc cells and MCF7 cells. Unlike the present HO-PBDEs, these HO-PCBs possessed tri- to tetrachlorine substitution on the phenolic ring. In this study, only three of the PBDEs [2,2´,4,4´,5,5´-hexaBDE (BDE-153), 2,3,4,4´,5,6-hexaBDE (BDE-166), and 2,3,3´,4,4´,5,6-hepta-BDE (BDE-190)] showed antiestrogenic activities with concentrations resulting in 50% inhibition (IC_{50} values) ranging from 0.8 to 3.1 µM. These PBDEs are likely not metabolized *in situ* because the congeners are hexa- or heptabromine substituted, have two *para*-bromines, and have no adjacent or *ortho-meta* brominated carbons. Since the T47D.Luc cells express a functional Ah receptor, it may be possible that the anti-estrogenicity of these PBDEs is Ah receptor-mediated, as is the case for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and several other antiestrogens (*52*). BDE-153, -166, and -190 induced the highest maximal luciferase activity in an Ah receptor CALUX assay based on H4IIE.Luc cells, among the same set of 17 PBDEs (*21*).

The antiestrogenicity of Ah receptor ligands is directly correlated to their affinity for the Ah receptor and their CYP1A-inducing potency (*52*). As shown for TCDD-treated MCF7 cells (*53*), the result is enhanced estrogen catabolism, and lower availability of estrogen to the cell. This correlation between structure-antiestrogenicity– and structure-CYP1A–inducing potency has been shown for various halogenated aromatics such as TCDD and non-*ortho* PCBs *in vivo* and *in vitro* (*55,56*). The exact mechanism of antiestrogenicity is probably specific to species, cell type, and the estrogen-responsive gene. Other possible cellular mechanisms of Ah receptor-mediated antiestrogenicity of BDE-153, -166, and -190 may be that the Ah receptor decreases the binding of the ER to the estrogen-responsive element, or the Ah receptor could act as a repressor by inhibiting the binding of other transcription factors (ER) or the disruption of promotor function.

Interestingly, the HO-PBDEs induced luciferase to a higher maximum activity than the maximum induction generated by E_2, though at higher concentrations. This has been shown for several other compounds mimicking the natural estrogen in reporter gene assays. Legler et al. (*31*) reported this phenomenon for the environmental estrogens genistein, nonylphenol, bisphenol A, *o,p´*-DDT, and methoxychlor in the same T47D.luc cells. Routledge and Sumpter (*57*) showed that genistein and 4-*tert*-octylphenol

induced luciferase activity at a higher level than estradiol in a recombinant yeast strain. The mechanism of this high induction is not yet resolved, but effects on luciferase stability or stimulation of the expression of the receptor or co-activation factors are hypothesized to be involved (*31*).

We detected no striking differences in the relative binding affinities for the tested compounds between ERα or ERβ. However, the agonistic activity compared to E_2 of BDE-30 and BDE-100 was much higher in the 293-ERα- than in the 293-ERβs-Luc cell line (Figure 6). Moreover, the agonistic activity of T_2-like HO-BDE, but not 4-phenoxy-phenol, was estrogen-receptor dependent (Figure 6). The induction of luciferase compared to E_2 by T_2-like HO-BDE was much higher in the 293-ERα-Luc assay, whereas the induction of luciferase by 4-phenoxy-phenol was not selective to either assay. This would suggest that the presence of a bromine atom adjacent to the phenolic hydroxyl group is a disciminating factor leading to a partial agonistic activity in the 293-ERβs-Luc cell line compared to a full agonistic activity in the 293-ERα-Luc cell line.

In the same two ER-CALUX assays, polycyclic musk compounds were selective to the 293-ERα-Luc but not the 293-ERβs-Luc assay (*32*). HO-PCBs with chlorine atoms only on the nonphenolic ring were found to bind with purified human ERα and ERβ with at least a 10-fold greater affinity than HO-PCBs with chlorine atoms on the phenolic ring (*34*). However, the binding preference was 2-fold greater for the ERβ over the ERα. In the same study, 4-HO-2´,4´,6´-trichlorobiphenyl and 4-HO-2´,3´,4´,5´-tetrachlorobiphenyl highly induced luciferase activity in transiently transfected 293-ERα-Luc and 293-ERβs-Luc cells, although the transactivation activity was higher in the 293-ERα-Luc cells.

In conclusion, the results from this study clearly demonstrate that several pure PBDE congeners, but especially hydroxylated PBDEs and polybrominated bisphenol A compounds, induce the estrogen receptor signal transduction pathway *in vitro*. The estrogenic potencies of these flame retardants are in the same range as the well-known environmental estrogen bisphenol A. The structure–activity relationships of the PBDEs are in accordance with structure–activity relationships proposed for hydroxylated polychlorinated biphenyls. Further, the agonistic potency *in vitro* of estrogenic PBDEs and HO-PBDEs is preferential toward the ERα relative to ERβ. Because of the high-production volume of these compounds and their accumulation in the environment, further studies on the possible implications of these findings for the *in vivo* situation are necessary.

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